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STUDIES ON THE EFFECT OF VARIOUS ADDITIVES ON THERMOSTABLE PROTEASE EXTRACTED FROM *ZINGIBER OFFICINALE* RHIZOME

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ABSTRACT

Zingiber officinale is one of the richest sources of proteolytic enzymes; which further have great potential in industries. In the present study, protease extracted from *Z. officinale* has been characterized biochemically with respect to various additives viz; metal ions, detergents and organic solvents. The pH stability of protease ranged from 4.0 to 6.0 and exhibited thermostability upto 50^0C. Out of the cations tested in the form of their respective salts; K^+>Na^+>Zn^{2+}>Cu^{2+} were found have stimulatory effect ranging upto 15% in descending order. On the contrary, Mn^{2+}>Co^{2+}>Fe^{3+} inhibited the protease activity. Mn^{2+} showed maximum inhibitory effect upto 35% showing its great potency. On treatment with various organic solvents (0.1%), all except toluene inhibited the protease activity upto 30%. Out of detergents tested at 0.1%, Tween-80 acted as strongest inhibitor showing upto 80% decrease in activity followed by Tween-20 (40% inhibition) while Triton X-100 and Sodium Lauryl Sulphate (SLS) acted as activators.

KEYWORDS: **Protease, Inhibitors, Activators, *Zingiber officinale*, Thermostable, Additives**

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INTRODUCTION

Ginger, known botanically as *Zingiber officinale* Roscoe, has its origin in South Asia but has spread to many other regions of the world, where it is used in foods or other applications. In the ancient times, ginger was used as medicine in India, China and Europe. Today, ginger is one of the most important and widely used spices in the world for treatment of diarrhea, cold and as appetite stimulant.[1] Ginger rhizome is also used in pharmacy due to the of the phenolic substance such as gingerol and shagaol which was reported to have anti-cancer and antioxidant activities.[2, 3] Proteases are a unique class of enzymes as they possess both degradative and synthetic properties.[4] They are of immense physiological as well as commercial importance. Therefore, researchers keep searching for new sources of plant protease to meet industrial needs. Hence, Ginger is of considerable interest as a potential new source of protease for industry. Ginger rhizome has been found to contain proteolytic enzymes reported by Thompson *et al.* in the year 1973. Cysteine proteases have been isolated from ginger (*Z. officinale*) rhizome in the past.[5] Amongst all the plant protease ginger protease is one of the least studied of the plant thiol proteases including papain, bromelain, ficin and actinidin.[6, 7] Ginger protease is an active meat tenderizer against collagen and other connective tissue proteins [8-11] In order to promote the commercial utilization of ginger protease on food and other industry, it is important to characterize the enzyme from different varieties of ginger.

MATERIALS AND METHODS

Z. officinale was obtained from local market. All chemicals were of reagent grade and obtained from standard commercial firms.

Extraction of protease enzyme from Z. officinale

The pre-weighed samples were crushed in sodium acetate buffer (pH 5.0, 0.05 M), filtered through Whatman filter paper and centrifuged for 20 minutes at 10,000 rpm at 4^0C. The pellet was discarded, supernatant collected and used for biochemical characterization.

Effect of pH on protease activity and stability:

The effect of pH on protease activity was measured in the pH range of 3.0-10.0, using appropriate buffers at concentration of 1 mM under standard assay conditions. To study stability as a function of pH, 100 µL of the crude enzyme was mixed with buffer solutions and incubated at 30^0C for 2 h and then aliquots of the mixture were taken to measure the residual protease activity (%) under standard assay conditions.

Effect of temperature on protease activity and stability

The influence of temperature on activity of protease was studied by incubating the reaction mixture at 30^0C time period ranging between 10-90 minutes and the product released estimated by Folin's method. The enzyme was incubated at different temperatures 10^0C-90^0C for 2 h to study the stability of the enzyme. The residual protease activity was measured by conducting the reaction at temperature 30^0C and pH 5.0. The activity of the enzyme was considered as 100% under standard assay conditions.

Effect of metal ions on protease activity

The effect of different metal ions on protease activity was determined by the addition of metal ions such as Cu^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Na^{+}, K^{+} in the form of respective salts i.e. $CuSO_4$, $CoCl_2$, $MnCl_2$, $FeCl_3$, $ZnSO_4$, NaCl and KCl at the final concentration of 0.25 mM. The effect was assessed by comparing with additive blank as control.

Effect of organic solvents on protease activity

The influence of various organic solvents on the activity of protease was studied in the presence of acetone, propanol, ethanol, methanol, acetone, toluene and butanol at the concentration level of 0.1% . The residual protease activity was estimated against control (organic solvent blank) and expressed in terms of percentage.

Effect of surfactants on protease activity

The effect of anionic surfactant (SLS) and nonionic surfactants (Tween-20, Tween-80, Triton X-100) on activity of protease from *Z. officinale* was investigated at 0.1% into reaction mixture and activity determined. By referring the control without chemical agents the residual activities were determined and expressed as relative values.

Statistical analysis

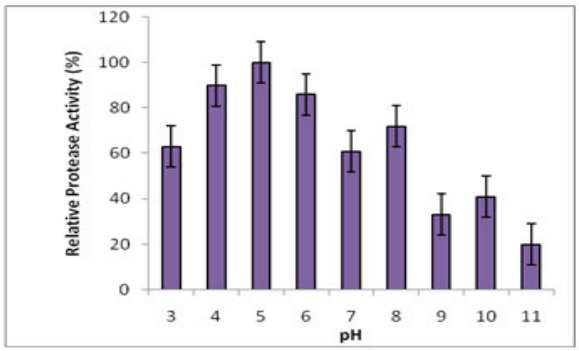
The results were subjected to *t* –test to determine P value by using GraphPad software.

RESULTS

Effect of pH on protease activity and stability

The pH optimum of protease from *Z. officinale* was found to be 5.0 (Figure 1). Enzymes, being pH sensitive get their activity affected by changing pH due to the charges on an amino acid residue which is functional in substrate binding or catalysis.[12]

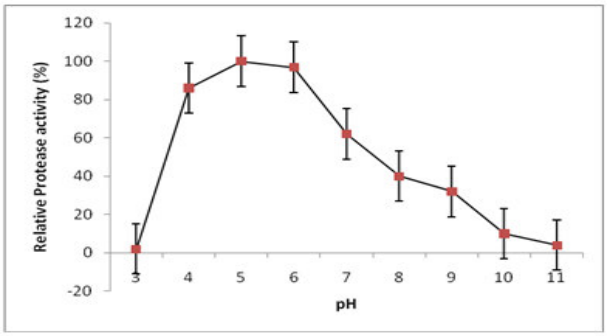
Figure 1
pH profile of protease isolated from Zingiber officinale



Mean Error bars in the figure represnts the mean ± standard error from the triplicate samples that were tested.
The two-tailed P value is less than 0.0001. By conventional criteria, this difference is considered to be extremely statistically significant.

Similar observation was reported in metalloprotease production in *P. fluorescens* and *Adhatoda vasica* with an optimum activity at pH 5.0.[13,14] The protease produced by isolates with enzymatic activity optima at pH 5.0 can be used to coagulate milk proteins for the dairy industry, as debittering agents in cheese and in peptide synthesis.[15] Figure 2 shows the pH stability curve of protease from *Z. officinale*. The protease was found to retain its activity in the pH range of 4.0 to 6.0.

Figure 2
pH stability curve of protease isolated from Z. officinale

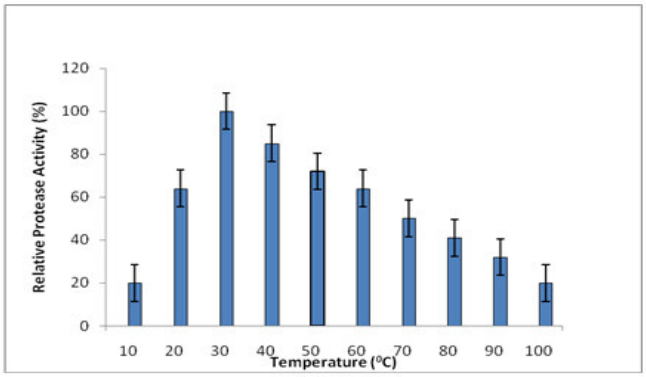


Mean Error bars in the figure represnts the mean ± standard error from the triplicate samples that were tested.
The two-tailed P value equals 0.0021. By conventional criteria, this difference is considered to be very statistically significant.

Effect of temperature on protease activity and stability

The temperature optima of protease isolated from *Zingiber officinale*, showing maximum activity at 30^{0}C (Figure 3). Increase in temperature above optimum level affects important factors like protein denaturation, protein ionization state and solubility of species in solution reducing enzyme activity.[16] Figure 4 depicts the thermal stability curve of the isolated protease showing activity retention up to 50^{0}C upto 2h of incubation period.

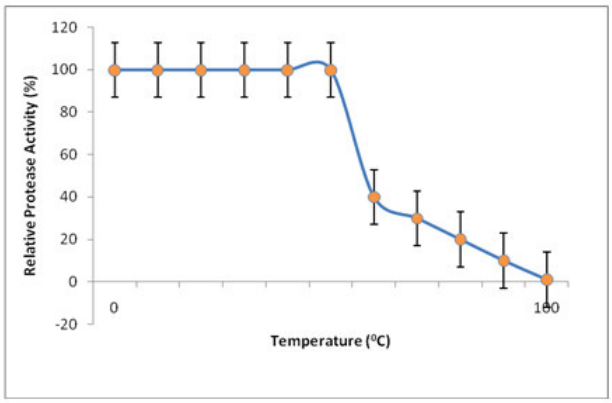
Figure 3
Temperature optima of protease isolated from Z. officinale



Mean Error bars in the figure represnts the mean ± standard error from the triplicate samples that were tested.
The two-tailed P value equals 0.0001. By conventional criteria, this difference is considered to be extremely statistically significant.

Proteases from other sources with temperature stability upto 50^{0}C have been reported by various workers. [17-21]

Figure 4
Thermostability curve showing heat resistancepattern of protease isolated from Z. officinale



Mean Error bars in the figure represnts the mean ± standard error from the triplicate samples that were tested.
The two-tailed P value equals 0.0011. By conventional criteria, this difference is considered to be very statistically significant.

Effect of metal ions on protease activity

The monovalent, divalent and trivalent cations (in form of respective salts) such as $CuSO_4$, $CoCl_2$, $MnCl_2$, $MnSO_4$, $FeCl_3$, $ZnSO_4$, NaCl and KCl were tested. K^+>Na^+>Zn^{2+}>Cu^{2+} were found having stimulatory effect ranging upto 15% in the order depicted in Table 1. On the other hand Mn^{2+}>Co^{2+}>Fe^{3+} inhibited the protease activity in the descending order. Mn^{2+} showed maximum inhibitory effect upto 35% showing its great potency. Results were recorded as the percent residual activity calculated with reference to activity of controls incubated in the absence of the additives used in this study.

Table 1
Effect of metal ions on activity of protease isolated from Z. officinale The activity of the enzyme without metal ion was taken as 100%.

S. No.	Metal ions	*Residual protease activity (%)
1.	Control	100
2.	$CuSO_4$	102
3.	$CoCl_2$	92
4.	$MnCl_2$	65
5.	$FeCl_3$	96
6.	$ZnSO_4$	111.39
7.	NaCl	114.53
8.	KCl	115
9.	$MnSO_4$	80.2

All results are mean of triplicate readings.

Protease activity was stimulated by Cu^{2+}, Zn^{2+}, Na^+ and K^+ indicating that these ions had a functional role in the molecular structure of the enzyme. Mn^{2+}, Co^{2+} inhibit the protease activity in coherence to the observation of Gupta *et al.* (2005) and Rahman *et al.* (1994).[22, 23] Fe^{3+} was found to decrease the protease activity in studies reported by Pooja and Gurunathan (2011). [24]

Effect of organic solvents

Proteases from *Z. officinale* had the ability to act in the presence of 0.1% concentration of organic solvents in the reaction system. Out of the six organic solvents tested toluene; stimulated the enzyme activity by 2% while other organic solvents reduced the activity ranging from 5% to 30% (Table 2).

Table 2
Effect of various organic solvents on activity of proteaseisolated from Z. officinale. The activity of the enzymewithout organic solvent (control) was taken as 100% (control).

S. No.	Metal ions	Residual protease activity (%)
1.	Control	100
2.	Ethanol	95
3.	Propanol	92
4.	Toluene	102
5.	Methanol	71
6.	Acetone	75
7.	Butanol	66

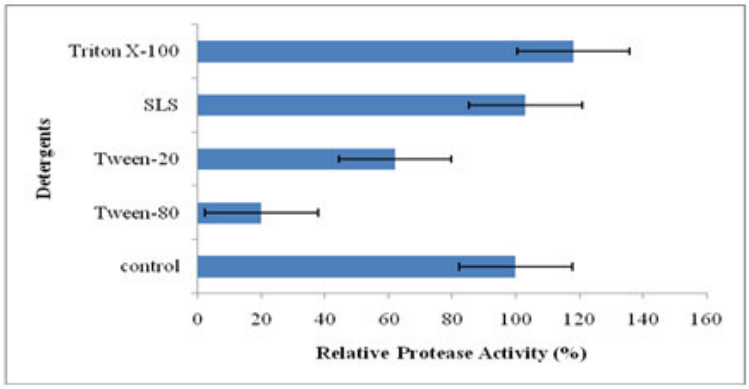
****All results are mean of triplicate readings.***

Effect of detergents

The effect of detergents on the activity of ginger protease is depicted in Figure 5. The emulsifiers such as Tween-20 and Tween-80 based on the 0.1% concentration decrease the activity of the ginger protease upto 40% to 80% respectively.[25]

Figure 5

Effect of various detergents on activity of protease isolated from Z. officinale. The activity of the enzyme without detergent (control) was taken as 100% (control).



Mean Error bars in the figures represnts the mean ± standard error from the triplicate samples that were tested.
The two-tailed P value is less than 0.0001. By conventional criteria, this difference is considered to be extremely statistically significant.

Triton X-100 and SLS as non-ionic and ionic detergent, respectively, enhanced the activity of ginger protease slightly.[26,27] Detergents such as Triton X-100 are often used in drug discovery research to weed out small molecule promiscuous and non-specific inhibitors which act by aggregation in solution and undesirable precipitation in aqueous assay buffers.

DISCUSSION

In the present investigation, protease enzyme extracted from *Z. officinale* has been biochemically characterized with respect to pH and temperature optimum conditions along with stability. The pH stability of protease ranged from 4.0 to 6.0 and exihibit thermostability upto 50^0C, in coherence with few other studies.[17-21] The effect of various additives; metal ions, organic solvents and detergents have been studied. Out of the cations tested in the form of their respective salts; K^+>Na^+>Zn^{2+}>Cu^{2+} were found have stimulatory effect ranging upto 15% in descending order. On the contrary, Mn^{2+}>Co^{2+}>Fe^{3+} inhibited the protease activity. Mn^{2+} showed maximum inhibitory effect upto 35% showing its great potency. Mn^{2+}, Co^{2+} and Fe^{3+} have been reported as inhibitors by few other workers.[22-24] On treatment with various organic solvents (0.1%), all except toluene inhibited the protease activity upto 30%. According to Najafi *et al*. in 2005, this capability could be due to disulfide bonds.[28] There are a number of reports on the importance of disulfide bonds for the stability of proteins in the presence of solvents. The proteases studied here could be very useful for fermentation and other reactions in the presence of solvents. One of the most important advantages of this property is to reduce or abolish the microbial contamination during the degradation reactions. Enzyme-catalysed reactions in organic solvents have found numerous applications, some of which have been commercialized.[29] There is great industrial demand for organic solvent stable proteases to employ in the synthesis of useful pharmaceutical products.[30] Over the years, intensive research has led to a variety of approved protease inhibitors for the treatment of various diseases.[31] Therefore, proteases, which are naturally stable in organic solvents, are essential for synthetic reactions and peptide synthesis. However, further research is needed to elucidate the effect of surfactants on the rate and extent of enzyme turnover.Triton X-100 and Sodium Lauryl Sulphate (SLS) acted as activators. Out of the detergents tested at 0.1%, Tween-80 acted as strongest inhibitor showing upto 80% decrease in activity followed by Tween-20 (40% inhibition). This reduction may be due to change in the enzyme confirmation when its inner site is distributed by non-polar site of those detergents.[26] The amphipathicity of the surfactant may also play a role in exposing the active sites available for enzyme substrate hydrophobic interaction.[32] The proteolytic activity of ginger rhizome was studied with casein as substrate. The above facts confers that the protease from *Z. officinale* is thermostable with great potential for future research with trade and industry feasibility. Thermal stability increases the efficiency of enzymes and is one of the essential features for their commercial exploitation. Further, it can be used to for making new milk curd products. The effect of metal ions suggests that metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at higher temperature. Triton X-100 may also improve the thermal stability of the enzyme thereby, improving the industrial usefulness. Based on its stability and activity profile, ginger protease can act as excellent lead for applications where resistance to harsh process conditions is required. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.[33]

CONCLUSION

Although there are many potent protease sources are on market for enzyme production, researchers prefer studying on new isolates because they could be alternative for commercial use. In the present investigation, *Z. officinale* rhizome has been found to be viable source of protease which is theromostable with great potential for future research with economic feasibility. It can be used to coagulate milk proteins for the dairy industry, as debittering agents in cheese, peptide synthesis, meat tenderizer, in addition to other industrial applications. Hence, based on the biochemical properties and effect of various additives, evaluated in this study, the protease extract from *Z. officinale* rhizome appears to be a promising candidate in making it particularly attractive for various biotechnological applications.

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